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*J Immunol* 2014; 193:2743-2752; Prepublished online 11 August 2014;
doi: 10.4049/jimmunol.1302338
http://www.jimmunol.org/content/193/6/2743

Supplementary Material  http://www.jimmunol.org/content/suppl/2014/08/09/jimmunol.1302338.DCSupplemental

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Hepatocyte Growth Factor Limits Autoimmune Neuroinflammation via Glucocorticoid-Induced Leucine Zipper Expression in Dendritic Cells

Mahdia Benkhoura, Nicolas Molnarfi, Isabelle Dunand-Sauthier, Doron Merkler, Gregory Schneider, Stefano Bruscoli, Carlo Riccardi, Yasuhiko Tabata, Hiroshi Funakoshi, Toshikazu Nakamura, Walter Reith, Marie-Laure Santiago-Raber, and Patrice H. Lalive

Autoimmune neuroinflammation, including multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), a prototype for T cell–mediated autoimmunity, is believed to result from immune tolerance dysfunction leading to demyelination and substantial neurodegeneration. We previously showed that CNS-restricted expression of hepatocyte growth factor (HGF), a potent neuroprotective factor, reduced CNS inflammation and clinical deficits associated with EAE. In this study, we demonstrate that systemic HGF treatment ameliorates EAE through the development of tolerogenic dendritic cells (DCs) with high expression levels of glucocorticoid-induced leucine zipper (GILZ), a transcriptional repressor of gene expression and a key endogenous regulator of the inflammatory response. RNA interference–directed neutralization of GILZ expression by DCs suppressed the induction of tolerance caused by HGF. Finally, adoptive transfer of HGF-treated DCs from wild-type but not GILZ–deficient mice potently mediated functional recovery in recipient mice with established EAE through effective modulation of autoaggressive T cell responses. Altogether, these results show that by inducing GILZ in DCs, HGF reproduces the mechanism of immune regulation induced by potent immunomodulatory factors such as IL-10, TGF-β1, and glucocorticoids and therefore that HGF therapy may have potential in the treatment of autoimmune dysfunctions.


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strain the Ag-presenting function of mouse DCs (7) and to govern the development of Treg cell–inducing regulatory DCs (11, 12). We recently suggested that such a mechanism might account for the protective role of CNS-restricted overexpression of HGF in myelin oligodendrocyte glycoprotein (MOG)–induced EAE (11). From an immunologic and therapeutic standpoint, it is important to understand the molecular basis involved in DC immunoregulation by HGF.

In addition to protective autoimmune, HGF plays functional roles in the CNS. HGF acts as a neurotrophic factor for a variety of neurons and exerts neuroprotective and regenerative effects in various animal models (13–15). All of these properties may underlie the recently reported clinical benefit of human mesenchymal stem cell therapy through the HGF–c-Met pathway in immune- and nonimmune-mediated demyelination models (16). Consistent with these properties, recent advances in humans indicate that HGF levels increase in MS patients during immunomodulatory drug therapy (17) and may play an additional beneficial role in MS by stimulating mechanisms that govern the repair of damaged tissue (18).

In the current study, we identify a central role for DCs in mediating the immunomodulatory effects of HGF in MOG-induced EAE, an acute T cell–mediated attack of the CNS that results in chronic functional deficits. Using biodegradable microspheres for the controlled release of HGF, systemic treatment with HGF reduced disease severity and induced tolerance in the DC population. HGF-treated DCs were characterized by increased programmed death ligand 1 (PD-L1) expression and ability to promote the development of IL-10–secreting Treg cells. In DCs, HGF induced glucocorticoid-induced leucine zipper (GILZ), the most prominent mediator of glucocorticoid-induced immunosuppression (19). RNA interference–directed inhibition of GILZ expression by DCs suppressed the induction of tolerance caused by HGF. In adoptive transfer studies, HGF treatment of wild-type (WT) DCs, but not GILZ gene–deficient DCs, potently mediated functional recovery in mice with established EAE. Because DCs play an important role in inciting CNS autoimmunity, the identification of GILZ signaling in mediating the immunoregulatory effect of HGF on DCs furthers our mechanistic understanding of the protective role of HGF in autoimmune neuroinflammation.

Materials and Methods

Mice

C57BL/6 (H-2b) female mice were purchased from Janvier Laboratories (France). 2D2 mice in a C57BL/6 background expressing a TCR specific for the MOG53–55 (MOG) peptide were kindly provided by V.K. Kuchroo (Harvard University, Boston, MA). C57BL/6 GILZ knockout (KO) (20) and littermate control mice were provided by C. Riccardi (University of Perugia, Perugia, Italy). Mice were kept in accordance with the guidelines of the Geneva University School of Medicine (protocol number 1005/3741/3).

Induction and evaluation of EAE

Mice were immunized s.c. in the flanks with 100 μg MOG peptide (MEVGWYRSPFVRHLYRNGK; Anawa Trading) in complete CFA (DIFCO Laboratories), and 300 ng pertussis toxin (Sigma-Aldrich) in PBS (control) or PBS containing hrHGF (5 mg/ml) for 24 h at 4°C for complete absorption. Animals received a single s.c. injection (100 μl) of gelatin hydrogel microspheres with hrHGF (100 μg) or vehicle.

Purification of mouse splenic CD4+ T cells and CD11c+ DCs

Mouse splenic CD4+ T cells were negatively selected using an anti-mouse CD4+ T cell isolation kit (Miltenyi Biotec). To obtain DCs, spleens were minced and incubated with DNase I (1 mg/ml) and Liberase HT (Roche) (0.5 mg/ml) for 15 min at room temperature. Cold EDTA was added to a final concentration of 20 mM, and cell suspensions were incubated for 5 min at room temperature before filtering through nylon mesh to remove tissue and cell aggregates. Highly pure splenic DCs were subsequently positively selected using anti-mouse CD11c colloidal superparamagnetic microbeads (Miltenyi Biotec), as reported previously (11). The purity of CD4+ and CD11c+ cells, confirmed by flow cytometry, was routinely >95 and >85%, respectively.

In vitro T cell and DC coculture assays

Splenic DCs (1 × 10^5 cells/ml) were pulsed with MOG (20 μg/ml) for 24 h and then cocultured in medium with TCR MOG CD4+ (2D2) T cells (1 × 10^5 cells/ml) for 72 h. In certain experiments, splenic CD11c+ cells were isolated from nonpathological WT or GILZ KO mice or, at the time of sacrifice, from EAE mice that received gelatin hydrogel microspheres incorporating hrHGF (100 μg) or vehicle.

T cell proliferation assay

For Ag-specific restimulation of mouse T cells, 2 × 10^5 splenocytes were cultured for 3 d in the presence of MOG. Cells were isolated from MOG-immunized mice at the time of sacrifice by homogenizing spleens through a cell strainer (70-μm nylon, Falcon; BD Biosciences) and removing RBCs in cold 0.83% NH4Cl/0.01 M Tris-HCl (pH 7.2). Proliferation was measured by incorporation of [3H]methylthymidine (1 μCi/well) during the last 16 h of culture using a filtermate harvester (Packard Instrument) and a 1450 liquid scintillation counter (PerkinElmer). DNA was extracted and analyzed as a percentage of total DNA. Alternatively, CD4+ T cell proliferation was measured by quantifying Ki-67 staining (BD Pharmingen) by flow cytometry. In some experiments, purified CD11c+ cells from WT or GILZ KO mice were cultured with MOG and naive 2D2 CD4+ T cells.

Adoptive transfer of DCs

CD11c+ cells of up to 95% purity were isolated from spleens of 8–10-wk-old C57BL/6 GILZ KO or WT littermate mice by positive MACS (Miltenyi Biotec). Purified CD11c+ cells (1 × 10^6 cells/ml) were cultured alone or with HGF (30 ng/ml) for 24 h, followed by a 4-h pulsing period with MOG (20 μg/ml), and immediately transferred into recipient mice. Recipient C57BL/6 mice were immunized with MOG, randomized at EAE score 1 or 2, and injected i.v. with 1 × 10^6 HGF- or vehicle-treated MOG-pulsed DCs.

RNA-mediated interference

GILZ gene expression silencing in DCs was obtained with a mixture of 20 nmol small interfering RNA (siRNA) specific for GILZ mRNA (siGILZ; Microsynth) and X-tremeGENE siRNA transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions.

ELISA

IL-12p70, IL-12p40, IL-27, IL-10, IL-6, IL-17A, IFN-γ, GM-CSF, murine HGF (mHGF), and hHGF were detected in culture supernatants using ELISA sets from eBioscience or R&D Systems according to the manufacturer’s instructions. Serum was isolated after coagulation and centrifugation (5 min at 3000 × g) and kept frozen at −20°C until use. For measurement of tissue HGF concentration, spleen from mice was ho-
mogenized in an extraction buffer containing 20 mmol/l Tris-HCl (pH 7.5), 2 mol/l NaCl, 0.1% Tween-80, 1 mmol/l EDTA, and 1 mmol/l PMSF. After centrifugation (19,000 × g for 20 min at 4°C), the supernatant was recovered for determination of mHGF and hHGF. Total protein levels were determined using a Bradford assay (Sigma-Aldrich) with BSA as a standard. The concentration of HGF in organs was expressed as nanograms per milligram total protein.

Isolation of CNS-infiltrating mononuclear cells

At the time of sacrifice, mice were perfused through the left cardiac ventricle with cold PBS. Spinal cords were extruded by flushing the vertebral canal with cold PBS and minced with a scalpel blade. The spinal cords were forced through 100-mesh stainless steel screens (Falcon; BD Biosciences) to obtain a single-cell suspension in HBSS containing 300 U/ml per cord type IV clostridial collagenase (Sigma-Aldrich) and then incubated for 1 h at 37°C. Infiltrates were isolated on Percoll (GE Healthcare).

Histopathology and immunostaining

Spinal cords obtained from transcardially perfused mice (4% paraformaldehyde) at the sacrifice time (day 21 postimmunization) were embedded in paraffin (n = 3 animals/experimental group). Paraffin-embedded blocks were cut into sections (3 μm thickness) and stained with H&E to assess inflammation or Luxol Fast Blue/periodic acid-Schiff stain to assess the degree of demyelination. Quantification was performed by examining 11 transverse sections from the cervical to lumbar spinal cord. The number of lesions per spinal cord section was averaged to calculate the inflammatory index. Extent of demyelination was expressed as percent of total white matter analyzed (set as 100%).

Immunologic markers and flow cytometry

Single-cell suspensions from spleens and spinal cords were incubated in blocking solution (PBS with 1% FCS) for 20 min on ice prior to staining to block nonspecific Fc-mediated interactions and then stained for 30 min at 4°C with FITC, PE, PerCP-Cy5.5, or allophycocyanin fluorochromes conjugated with Abs (1:100) against: CD11c, MHC class II, CD40, CD86, c-Met, PD-L1, GILZ, CD4, CD44, CD62L, and CD25 (all Abs from eBioscience) or appropriate fluorochrome-conjugated, isotype-matched irrelevant Abs to establish background fluorescence. For intracellular cytokine staining of IFN-γ, IL-17, and IL-10 (eBioscience), T cells were stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich) and then fixed and permeabilized using BD Cytofix/Cytoperm Plus Kit (BD Biosciences). Foxp3 staining was performed according to the manufacturer’s protocol (FJK-16s; Mouse Treg Cell Staining Kit; eBioscience). Samples were processed on an FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo analysis software (version 9.3.2; Tree Star).

Western blotting

Purified splenic DCs cultured in vitro in different conditions were homogenized using a polytron in lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM DTT) containing complete protease inhibitors (Roche). Equal amounts (20 μg) of total protein from each sample were transferred to a 15% SDS-polyacrylamide gel and blotted onto an Immobilon-P PVDF membrane (Millipore). GILZ protein was detected using properly diluted (1/100) rat anti-mouse anti-GILZ Ab (eBioscience), followed by a peroxidase-conjugated secondary Ab to rat IgG (Jackson ImmunoResearch laboratories) and then visualized using chemiluminescence (Supersignal; Pierce). The blot was also probed with β-actin mAb as a loading control (Sigma-Aldrich).

RNA isolation and real-time quantitative PCR

RNA was prepared from MACS-sorted DCs using Qiagen RNAeasy Mini Kits (Qiagen) and subjected to DNase I (Roche Diagnostics) digestion. Random hexamer primers (Promega, Madison, WI) and Superscript II (Invitrogen, Carlsbad, CA) were used to generate cDNA. GILZ transcripts were quantified by real-time PCR analysis using SYBR Green as the detection agent. The PCR was performed with the Bio-Rad iCycler iQ system (Bio-Rad). GILZ mRNA expression was normalized to β-actin expression and quantified using the relative standard curve method, followed by comparison with the results from control samples (unmanipulated DCs). For all reactions, each condition was performed in triplicate. Each experiment was repeated at least three times.

RNA-mediated interference

Freshly purified splenic DCs were incubated with a mixture of 20 nmol siGILZ (Microsynth) and X-tremeGENE siRNA transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. The siRNA sequence targeting mouse GILZ was as follows: 5′-GCAGGAUUUGAUUGUUAGAAUA-3′. Control cells were either mock transfected or transfected with 20 nmol nonsilencing scrambled control siRNA (sc) (5′-GACACGACGUUAGAACTT-3′ (Microsynth). At 24 h posttransfection, cells were divided into several dishes for stimulation and analysis. The transfection efficiency of siRNA was determined by flow cytometry with Alexa Fluor 488-labeled negative siC and routinely reached values >80%.

Serum corticosterone levels

Measurement of corticosterone in serum was performed by an enzyme immunoassay kit (Enzo Life Sciences, Lausen, Basel, Switzerland) according to the manufacturer’s instructions. The detection limit was 27 pg/ml. Serum was isolated after coagulation and centrifugation (5 min at 3000 × g) and kept frozen at −20°C until use.

Detection of anti-hrHGF Abs

Serum hrHGF-specific IgG was quantified using a noncommercial ELISA. The 96-Maxisorb plates (Costar) were coated with hrHGF protein (10 μg/ml in PBS), blocked with BSA (Sigma-Aldrich), and incubated with sera overnight at 1:250 dilution. After washing, hrHGF-specific IgG retained by the plate-bound hrHGF was detected with HRP-conjugated anti-mouse IgG (Immu-no-Tek). The positive control was performed with a mouse monoclonal anti-human full-length HGF Ab (R&D Systems) using 2-fold serial dilution (0.0315–0.5 μg/ml). BSA-coated plates were used as negative controls for nonspecific binding. SOFTmax ELISA plate reader (405-nm wavelength) and software (Molecular Devices) were used for data analysis.

Statistical analysis

All of the statistical analyses were performed by GraphPad Prism for Mac, version 5.0 (GraphPad).

Results

Controlled release of HGF attenuates development of MOG-induced EAE

Generally, i.v. injections of HGF exert only short-term effects on target organs and do not produce continuous effects (16). To achieve effective delivery of HGF, we selected biodegradable gelatin hydrogels as a carrier for hrHGF and administered the hrHGF/gelatin complex by s.c. injection (21). Without altering the concentration of endogenous mHGF, sustained and supraphysiologically relevant (3-fold) hrHGF levels in sera and spleens were detected in EAE mice for at least 10 d after s.c. injection of the gelatin/hrHGF complex (henceforth referred to as HGF) (Fig. 1A, 1B). HGF treatment significantly suppressed the severity of MOG-induced EAE without a reduction in disease incidence (Fig. 1C, Table I). Histological examination at the sacrifice time (day 21) demonstrated that HGF-treated mice had reduced CNS inflammatory infiltration and demyelination as compared with mice treated with vehicle (Fig. 1D). In addition, mice treated with HGF showed higher IL-10 (Fig. 1E, left panel) and lower IFN-γ (Fig. 1E, right panel) production from CNS-infiltrating CD4+ T lymphocytes compared with control mice at the latest time point. HGF enhances Treg cells and limits encephalitogenic T cell responses in EAE

To evaluate whether HGF treatment ameliorates EAE by modulating the peripheral T cell immune responses that are necessary for the development of the disease, inflammatory profiles of CD4+ splenocytes from mice treated with HGF or vehicle were analyzed. Spleen cells obtained at the sacrifice time from the HGF-recipient mice demonstrated significantly increased levels of CD44highCD62Llow naive CD4+ T cells (Fig. 2A, top panel), CD4+CD25+Foxp3+ Treg cells (Fig. 2B, top panel), and IL-10–producing Treg cells (Fig. 2B, bottom panel). Correspondingly, mice that received HGF treatment showed a reduced proportion of activated CD44highCD62Llow CD4+ T cells (Fig. 2A, bottom panel) and a 2-fold reduction in the frequencies of both IFN-γ and IL-17+ splenic CD4+ T cells (Fig. 2C). These results indicate...
that the downregulation of both Th1 and Th17 effector cells and the upregulation of Treg cells are associated with the clinical benefit of HGF on EAE pathogenesis. To measure primary recall T cell responses, spleen cells were restimulated with MOG. Splenic T cells obtained from HGF-treated mice demonstrated significantly reduced cell proliferation and production of IFN-γ, IL-17, and GM-CSF compared with cells from control mice (Fig. 2D).

**FIGURE 1.** In vivo HGF treatment significantly suppresses development of EAE. (A and B) The gelatin/hrHGF complex provided sustained delivery of hrHGF. The levels of mHGF and exogenous hrHGF were determined by ELISA from sera at different time intervals postinjection (A) and spleens (day 22) (B); mean ± SEM for five animals. **p < 0.01, ***p < 0.001 by Student *t* test. (C) HGF is effective in reducing EAE progression. Mice received single s.c. injection of gelatin/PBS (control; white squares; *n* = 10) or gelatin/hrHGF complex (HGF; dark squares; *n* = 10) 2 d before immunization (MOG). Data are shown as mean clinical score ± SEM for each day postimmunization. Mann–Whitney *U* test was used to compare the daily EAE scores (*p < 0.05, **p < 0.01). Results shown are representative of two independent experiments of *n* = 10 mice/group. (D and E) Mice selected for histologic examination and CNS CD4+ T cell cytokine profile evaluation had clinical scores at the time of sacrifice (day 21 postimmunization) that represented the mean for their group. (D) The HGF treatment group (mean score 2.5) had significantly fewer cellular infiltration and demyelination compared with the control group (mean score 3.5). Representative images of paraffin-embedded spinal cord sections from indicated groups stained for H&E (HE) or Luxol Fast Blue/periodic acid-Schiff stain (LFB/PAS) are shown. Scale bar, 50 μM (original magnification ×200). The average number of inflammatory lesions (D, bottom left panel) and extent of demyelination (D, bottom right panel) from 11 sections of spinal cord per mouse (*n* = 3/group) are presented as bar graphs; mean ± SEM. **p < 0.01 by Student *t* test. (E) HGF decreased pathogenic T cell numbers within the CNS. CNS CD4+ T cells from mice treated with hrHGF or PBS were tested for IFN-γ and IL-10 secretion. Representative histograms and Gmean values of each determination are shown (mean, *n* = 5). *p < 0.01 by Student *t* test.

Table I. Systemic HGF treatment reduces EAE severity and progression

<table>
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<tr>
<th>Disease Incidence</th>
<th>Mean Day of Onset</th>
<th>Total Score</th>
<th>Area Under the Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin/PBS</td>
<td>10/10</td>
<td>11.5 ± 1.1</td>
<td>239 ± 6.9</td>
</tr>
<tr>
<td>Gelatin/hrHGF</td>
<td>10/10</td>
<td>13.1 ± 1.1*</td>
<td>142 ± 7.3**</td>
</tr>
</tbody>
</table>

C57BL/6j mice received a single injection of gelatin/PBS (*n* = 10) or gelatin/hrHGF complex (*n* = 10) 2 d before immunization (MOG). The mean day of onset was calculated by averaging the first day of clinical signs of individual mice within each group. The total score was calculated by summing all the scores obtained on the clinical scale over the time frame being analyzed. The area under the curve (AUC) was calculated from the EAE clinical course for each mouse between days 0 and 21 postimmunization. Differences in the mean day of onset, total score, and AUC, were analyzed by Mann–Whitney *U* test (experiment versus treatment group) for the data in Fig. 1C. Each mean value was reported with the SD. Results are representative of two separate experiments (10 mice/group/experiment).

*p < 0.05, **p < 0.01.

**HGF redirects T cell immune responses by modulating APC functions**

Next, we explored the ability of HGF to mediate immune modulation by altering APC function. Highly pure DCs were isolated from EAE mice treated or not with HGF (30 ng/ml) and tested as APCs for the stimulation of naive MOG-specific TCR transgenic (2D2) T cells in the presence of MOG. Significant reductions in the level of 2D2 T cell proliferation (Fig. 3A) and the production of...
IFN-γ, IL-17, and GM-CSF (Fig. 3B) were observed when HGF-treated DCs were used as APCs. There was a corresponding decrease in the frequency of 2D2 T cells with an activated CD44^high CD62L^low phenotype (Fig. 3C, bottom panel) and a paralleled increased number of CD4^+ T cells that retained the naive phenotype (CD44^lowCD62L^high) (Fig. 3C, top panel) when cultured with DCs from HGF-treated mice. Moreover, after coculture with DCs from HGF-treated mice, significant increases were observed in the proportions of Treg cells (Fig. 3D) (4.1 ± 0.4, mean ± SEM; n = 3) and IL-10–producing 2D2 CD4^+ T cells (Fig. 3E) (3.6 ± 0.9, mean ± SEM; n = 3). Similar results were obtained when the different cell populations were calculated as absolute numbers (Fig. 3F).

**HGF induces regulatory DCs characterized by increased expression of PD-L1 and GILZ**

Because APCs directed T cell immune modulation in HGF treatment, we evaluated the cytokines produced by DCs of mice immunized with MOG. As described in Fig. 4A, DCs from HGF-treated EAE mice exhibited a tolerogenic phenotype, characterized by reduced cellular production of the proinflammatory-polarizing Th1 cytokines IL-12 (p70) and IFN-γ as well as decreased production of the IL-12/IL-23 p40 subunit. HGF treatment further showed increased production of the Th17 cell–inhibiting cytokine IL-27 by DCs with no effect on IL-6 production. In these conditions, IL-10 production by DCs was very low in each group of mice and did not allow appropriate comparison. Flow cytometric analysis, however, revealed that HGF treatment was consistently associated with significantly increased IL-10 and PD-L1 expression in DCs, whereas HGF did not affect the expression of MHC class II or CD86 (Fig. 4B). As previously demonstrated (11), DCs from HGF-treated EAE mice exhibited reduced CD40 expression (Fig. 4B). During the search for molecules enabling tolerization of DCs, we addressed the expression of GILZ, an important gene reported to promote the differentiation of DCs into Treg cell–inducing regulatory DCs. Flow cytometry analyses of splenic DCs from EAE animals treated or not with HGF revealed that in vivo HGF treatment increased GILZ protein expression in DCs (Fig. 4B), providing a potential molecular mechanism underlying the tolerogenic function of HGF in DCs.

**GILZ expression directs the induction of tolerogenic DCs by HGF in vitro**

Using adoptive transfer EAE experiments, we further established that in vitro treatment of DCs with HGF could limit the full development of encephalitogenic potential in autoreactive T cells, hence recapitulating the in vivo biologic action of HGF delivery in EAE (Fig. 5A). Mechanistically, HGF-treated DCs in vitro reduced MOG-reactive T cell proliferation (Fig. 5B) and induced greater frequencies of myelin Ag-specific IL-10–producing Treg cells (Fig. 5C). As a crucial mediator of the anti-inflammatory and immunosuppressive activity of glucocorticoids (GCs), we chose to
further investigate the functional significance of GILZ with respect to the immune-regulatory properties of HGF. Similar to the control treatment with dexamethasone, a potent GILZ inducer in DCs (22), HGF treatment induced high expression of GILZ mRNA (Fig. 6A) and protein (Fig. 6B) in DCs. Using in vitro cocultures in which DCs were used as APCs with naive CD4+ 2D2 T cells and Ag (MOG) under nonpolarizing conditions, (A) DCs from HGF-treated mice are poor T cell stimulators. The proliferative response of 2D2 T cells was determined by the number of Ki-67 positive CD4+ T cells using flow cytometry after 3 d in culture. Data are representative of three independent experiments (n = 3 mice/group). (B) DCs from HGF-treated mice limit T cell differentiation to encephalitogenic Th1 and Th17. IFN-γ, IL-17, and GM-CSF levels were quantified by ELISA. Bars represent the mean ± SEM of triplicate wells per mouse (n = 3/group). (C) T cells were also analyzed for the expression of CD44 and CD62L by flow cytometry (n = 3 mice/group). (D and E) DCs isolated from mice treated with HGF promote Treg cell differentiation. Intracellular cytokine staining shows the percentage of CD25+ Foxp3+ and IL-10+ CD4+ T cells after 3 d in culture (gate) (n = 3 mice/group). (F) Bar graphs show the absolute numbers, calculated by combining the cell concentration determination from the hematology analyzer (Neubauer cell count) with the flow cytometric population data of the different T cell populations in the culture systems. Error bars indicate SEM of triplicate wells per mouse (n = 3/group). *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.

Expression of GILZ by DCs is essential for HGF treatment to have protective effects in EAE

To confirm the involvement of tolerogenic DCs in EAE suppression by HGF and the importance of GILZ signaling in this mechanism, we performed adoptive transfer of DCs into recipient mice with established EAE (score 1). Highly pure splenic DCs isolated from nonpathological WT and GILZ-deficient mice were subjected to HGF treatment prior to adoptive transfer; control cells were not subjected to HGF treatment. Compared with the transfer of vehicle-treated control cells, adoptively transferred HGF-tolerized DCs from WT mice markedly blocked EAE progression and reduced clinical severity (Fig. 7A). Importantly, the adoptive transfer of HGF-treated DCs from GILZ KO mice did not alter EAE severity (Fig. 7A), which suggests that expression of GILZ in DCs is essential for the protection mediated by HGF against EAE. HGF-treated WT DCs also potently reduced EAE in mice with greater disease severity (Supplemental Fig. 1). Mechanistically, improvement of clinical EAE correlated with the capacity of HGF-treated DCs to significantly increase the percentage of Treg cells and IL-10–producing CD4+ T cells and to reduce that of both Th1 and Th17 effector cells (Fig. 7B). Complementary in vitro studies revealed that HGF-treated DCs from WT, but not GILZ KO mice, reduced the T cell proliferation (Fig. 7C) and differentiation of cytokine production associated with Th1 and Th17 cells (Fig. 7D). An expansion in the frequency of Treg cells was observed when we examined cultures using HGF-treated DCs from WT, but not GILZ KO, mice as APCs (Fig. 7E). DCs from WT and GILZ KO mice demonstrated similar surface expression of the HGF receptor c-Met.
Fig. 7F), ruling out the possibility that the defective tolerogenic effect of HGF on GILZ KO DCs was due to altered c-Met expression. Altogether, these data confirmed that by acting via GILZ, HGF is an important signal to prompt DCs to suppress T cell responses and promote tolerance.

**Discussion**

DCs are considered as the most potent APCs, capable of promoting Ag-specific immune responses in either an immunogenic or tolerogenic manner. Consequently, the identification of biological or pharmacological agents that promote tolerogenicity of DCs has
HGF LIMITS CNS AUTOIMMUNITY BY INDUCING GILZ IN DCs

received considerable attention regarding their application in the treatment of chronic immune-mediated conditions. Although the exact mechanism remains elusive, recent advances suggest that HGF can generate tolerogenic DCs capable of promoting the development of Treg cells with suppressive activity.

In this study, we examined the potential therapeutic properties of peripheral administration of HGF in the development and progression of MOG peptide-induced EAE, a prototypical animal model of T cell–driven autoimmune disease and, more specifically, of MS pathogenesis. We found that systemic HGF prevented autoimmune-mediated inflammation and demyelination and was associated with the development of DCs with a typical tolerogenic phenotype in vivo. We observed a significant increase in the frequency of both peripheral and CNS anti-inflammatory Treg cells and a concomitant decrease of proinflammatory Th1 and Th17 responses. This effect was independent of the action of HGF on the hypothalamic–pituitary–adrenal axis (Supplemental Fig. 2A), a crucial immunoregulatory pathway, or inhibition of DC activation via the formation of immune complexes (Supplemental Fig. 2B). Consistent with a role for immune tolerance induced by HGF, we demonstrated that HGF-treated DCs suppressed the encephalitogenic potential of myelin-reactive T cells to adoptively transfer EAE. These findings further validate that HGF-treated DCs on EAE. These findings further validate that HGF-treated DCs on EAE.

Importantly, we determined that HGF treatment significantly increases PD-L1 expression by DCs. Although these immunomodulating mechanisms may confer the regulatory properties of HGF-treated DCs, it does not provide the molecular mechanisms by which HGF generates tolerogenic DCs. Generally, immunomodulatory cytokines such as IL-10, or TGF-β1, or immunosuppressive agents such as GCs, used alone or in combination, promote the development of regulatory DCs (4). A common trait of tolerogenic DCs generated by IL-10, GCs, and TGF-β1 is the expression of GILZ (25). Expression of the GILZ molecule by DCs allows the induction of Treg cells and prevents DCs from activating Ag-specific T cells (22, 25). In this study, we show that HGF has a strong stimulatory effect on GILZ expression in DCs. Downregulation of GILZ by RNA interference fully restored Ag presentation by HGF-treated APCs, and GILZ deficiency abrogated the tolerogenic properties of adaptively transferred HGF-treated DCs on EAE. These findings further validate a major role for GILZ expression in determining whether DCs stimulate T cells.

HGF has been reported to ameliorate the progression of a number of inflammatory autoimmune diseases, including allergic airway inflammation (7), myocarditis (8), lupus nephritis (9), and arthritis (10), suggesting that HGF may interfere with a common proinflammatory process. The protective actions of HGF in all of these various organ system dysfunctions were tightly associated with the

FIGURE 6. HGF-induced GILZ expression converts immunogenic DCs to tolerogenic DCs. All experiments were conducted with highly pure splenic DCs isolated from naive (unimmunized) mice. DCs were subjected or not to in vitro HGF (30 ng/ml) or dexamethasone (DMX) (10−7 M) treatment for 24 h or as indicated in the figure. GILZ expression was examined by quantitative real-time PCR (A) and flow cytometric analyses (B). C–F) Inhibition of GILZ expression in DCs by siRNA prevents the generation of tolerogenic DCs by HGF. DCs were mock transfected without siRNA addition or transfected with siRNA duplexes directed against the coding region of GILZ (siGILZ) or with a nonsilencing siC. At 24 h after transfection, GILZ expression was determined by quantitative real-time PCR (C) and Western blotting analyses (D). (E and F) Transfected DCs were treated in vitro with HGF (30 ng/ml) or vehicle for 24 h and used as APCs in cocultures with naive CD4+ 2D2 T cells and MOG. GILZ knockdown deregulates the ability of HGF-treated DCs to maintain the size of the naive (CD44low CD25−) CD4+ T cell population (E, top panel) and to stimulate the generation of both CD25+Foxp3+ Treg cells (E, bottom panel) and IL-10–producing CD4+ T cells (F). Results are shown as representative flow cytometry contour plots and bar graphs (mean ± SEM of triplicate wells per mouse, n = 3/group). Data shown are representative of three independent experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001 by Student t test.
modulation of T cell immune responses, supporting a potent regulation of DC function. Mechanistically, HGF was reported to inhibit DC activation in vitro by disrupting NF-κB signaling via Bruton’s tyrosine kinase and a downstream signaling cascade involving sequential activation of the PI3K/AKT pathway and mammalian target of rapamycin (26). In this study, our in vitro and in vivo studies further delineate the molecular events by which HGF confers regulatory functions to DCs. Consistent with our findings, GILZ has been reported to modulate myeloid APC activation and central reactivation of myelin-specific T cells (2). In the EAE process, we have recently reported that selective HGF overexpression by neurons suppressed disease and was associated with increased frequencies of classical Treg cells and reduced production of IFN-γ and IL-12p70 within the spinal cord (11). Although no modulation in peripheral T cell activation was observed in this transgenic model, we demonstrate in this study that HGF reduces EAE by modulating peripheral T cell immune responses that are important for the development of the disease through the alteration of DC functions. Altogether, our studies thus indicate that HGF has the ability to ameliorate CNS autoimmunity by controlling the development of regulatory DCs in both the peripheral compartment and CNS tissues. Complementary to our findings, therapeutic administration of exogenously supplied HGF was recently reported to reduce functional deficits in mouse MOG-induced EAE and to promote the development of oligodendrocytes and neurons (16). Although the molecular and cellular mechanisms by which HGF affects immune responses were not addressed, our current findings suggest that induction of GILZ synthesis may potentially support HGF-mediated immunosuppression by regulating DC functions. Hence, in addition to the direct reported roles of HGF in neuroprotection and neurepair (16), the development of regulatory cells by HGF may confer indirect neuroprotection owing to the prevention of inflammatory damage.

The cumulative results of this study support the notion that Th1 and Th17 suppression with parallel elevation in Treg cells are central mechanisms underlying the immunomodulatory effect of HGF in experimental autoimmune neurinflammation. We estab-
lish that APCs are critical targets for the therapeutic effect of HGF and that stimulation of GILZ synthesis is a crucial mechanism of HGF action in DCs. Altogether, these results show that by inducing GILZ in DCs, HGF reproduces the mechanism of immune regulation induced by immunomodulatory factors and therefore that HGF therapy may have potential in the treatment of autoimmune dysfunctions such as MS.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


